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Selection of bacterial inoculants for ensiling and their effects on alfalfa haylage(1)

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Abstract:
The purpose of this study was to develop domestic inoculants to improve the silage quality of those materials with poor fermentation characters like legumes. Sixty strains of lactic acid bacteria were obtained from diverse resources including silage, pickled foods, yogurt, fruit, compost and animal manure. After evaluation of acid and salt tolerance, 22 strains were selected to screen their effects on alfalfa ensiling. In the preliminary screening, alfalfa of two different moisture contents was used and a commercial inoculant was used as positive control and no inoculant as negative control. After ensiling of 8 weeks, un-wilted alfalfa inoculated by strain ST15 had the lowest pH value of 4.61 and that of no inoculant control was 4.86. While in wilted condition, the set of strain ST12 had the lowest pH value of 4.88 and that of no inoculant control was 5.45. The strain D41 had the least gas production. Therefore, strains S15, S12 and D41 were used for further experiment. D41 was identified as *Lactobacillus casei*, and ST12 and sT15 were *L. plantarum*. In experiment II, the moisture content of alfalfa was wilted to 56.9% and ensiled with or without inoculant. Laboratory silos were opened and analyzed at 4, 8, 12 weeks for pH and fermentation products. The acetic acid and the lactic acid of no inoculant control ranged 0.29%-1.67% and 0.26%-0.69% while inoculant treatments ranged 0.29%-0.87% and 0.64%-2.24%, respectively. The results showed that inoculants produced more lactic acid and lowered acetic acid/lactic acid ratio than control. The Fleig's score of no inoculant control at 12 weeks reduced to 38, and those of inoculation treatment still maintained among 82 to 89. In addition, the Fleig's score of strain D41, ST12 and ST15 sets were higher than commercial strain (Ecosyl) set at 4 and 8 weeks, indicated that the domestic strains can improve the fermentation effectively.

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Introduction

Silage quality is affected by the type of crop, harvest period, moisture content, and the silage production process (Wilkinson, 1983). Additionally, the different fermented acids that are produced owing to differences in the microflora on the surface of the silage and the nutrient composition are important factors that affect the silage quality. The addition of lactic acid bacteria not only accelerates silage fermentation and enables silage to rapidly reach a stable state, but can also preserve its nutrient composition owing to the acidity (Kung, 1996; Kung and Ranjit, 2001)

High-protein forage has a high nutritional value, but plants have a high pH buffering capacity and low water-soluble carbohydrates, which are not factors that are conducive for silage fermentation. Therefore, an understanding of the crop maturity, moisture, and use of additives will assist in silage fermentation (McDonald *et al.,* 1991; Fraser *et al.*, 1998).

Many studies have also shown that the addition of lactic acid bacteria can increase silage quality (Oude Elferink *et al.*, 1997; Kung and Ranjit, 2001; Wang *et al*., 2008; Wang *et al*., 2009). In good silage fermentation, the pH value decreases with time and the pH changes between inoculated and uninoculated silage can be used as a marker to screen the microbial inoculants for silage (Jones *et al*., 1991; Stokes *et al.*, 1994).

Alfalfa is perennial legume forage, their symbiosis nitrogen fixation can play a relatively important role in forage cultivation systems. In temperate regions, alfalfa can be made into hay. In Taiwan, owing to climatic effects, only a low proportion of alfalfa is made into hay, and using haylage as a means of storage may be a viable option. However, it is still difficult to understand silage quality. Muck (1987; 1990) conducted alfalfa silage experiments and discovered significant protein degradation in period of wilting and ensiling processes, which resulted in an increase in plant buffering capacity and a limited reduction in pH. The adjustment of moisture content was a key factor to get good silage quality, whereas other conditions also had a large effect on silage quality. However, depending on the material characteristics, choice of proper pre-processing methods was important. The inoculation experiments of Filya et al. (2007) showed that silage quality was significantly improved by lactic acid bacteria inoculation.

The main aim of this study was to develop a local silage microbial inoculant to overcome the difficulty of legume forage preservation, and to increase nutrient content and self-sufficiency of domestic forage.

Materials and methods

I. Isolation and screening of bacterial strains

(i) Silage, pickles, yogurt, fruits, compost, and animal feces were used for the isolation of lactic acid bacteria. These materials were homogenized in sterile water, then serial decimal dilutions were obtained and plated on lactobacilli de Man, Rogosa, Sharpe (MRS) agar plates supplemented with 100 mg/mL cycloheximide. The plates were incubated in anaerobic condition at 30 °C for 48 h. To separate the pure lines, an isolated bacterial colony was selected, transferred into a fresh MRS plate, and the above steps were repeated three times. The purified bacterial strains were stored in 10% glycerol at -20°C. The preserved frozen bacterial strains were transferred into fresh MRS liquid medium, cultured for 24 h, and the pH values measured. The bacterial strains that produced acids at pH 4 and below were retained for Gram staining and microscopic examination. The retained gram-positive bacteria were continuously propagated for step 2 (bacterial strain tolerance testing).

(ii) The culture medium conditions were adjusted in order to screen for bacterial strains with a high adaptability to the environment: (1) Salinity tolerance testing: NaCl was used to adjust the salinity of MRS liquid medium to 10%, 5%, and 0% (no salt added) and the isolated strains were

inoculated into these media. After 48 h-incubation, the pH value of the media was measured. (2) Acidity tolerance testing: NaOH and HCl were used to adjust the pH of the MRS liquid medium to 3.5, 4.5, and 6.05. After inoculation for 24, 48, and 72 h, and the pH changes were measured.

II. Alfalfa silage

(i) Initial screening of bacterial strains

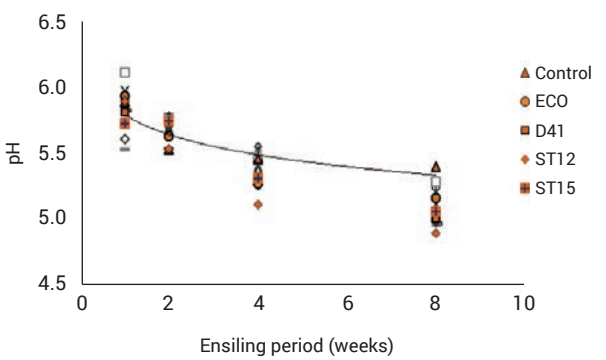
From the experiments on salinity tolerance and acidity tolerance, 13 and 9 bacterial strains with better growth were selected, respectively. A commercial strain of *Lactobacillus plantarum* (provided by Ecosyl products limited, ECO group) and a control treatment (with no inoculant) were added to give a total of 24 treatments in the initial screening comparison.

After harvest, alfalfa were divided into two sets, first set was cut and ensiled directly, the second set was wilt for 4 h then processed as the first set. Briefly, the materials were finely cut into pieces of 3–5 cm, mixed, and divided into 24 groups evenly for the 24 treatments. The inoculum amount was 1×10^6 cfu/g forage. After inoculation, the materials were evenly mixed and sealed in vacuum plastic bags, with each bag filled up to 135 g, and stored at room temperature. The bags were opened at different times and the pH values and voltaic fatty acids were measured to evaluate silage fermentation quality; one duplicate set was opened at each time point.

(ii) Comparison the effect of selected inoculants

The D41, ST12, and ST15 strains that were selected from the initial screening were sent to the Food Industry Research and Development Institute for identification and cultured in large amounts for subsequent experiment. After harvesting and wilting for 4 h, the moisture content of alfalfa was 56.9%. These wilted materials were mechanically cut into 3–5 cm sections for silage inoculation experiments and the materials were divided into five parts. One part was the control sample, which was not inoculated, and the remaining four parts were inoculated with D41, ST12, ST15, and the commercial strain ECO, respectively, with an inoculum volume of 1×10^6 cfu/g forage. After inoculation, the materials were evenly mixed and sealed in vacuum plastic bags, with each bag filled up to 120 g and stored at room temperature. The bags were unsealed at weeks 4, 8, and 12 to determine the silage fermentation status and nutrient content; one duplicate set was opened at each time point.

Fig. 1. Preliminary evaluation of the effects of isolated strains on pH value of alfalfa silage



III. Silage quality analysis

For acidity testing, 20 g fresh silage was mixed with 180 mL distilled water. After homogenizing and filtering, the acidity was measured using a pH meter. The gas chromatography method of Jones & Kay (1976) was applied for the quantitation of lactic acid, butyric acid, propionic acid, and acetic acid. The above silage extracts were passed through a cation column and the eluate was titrated with 0.05 N tetrabutyl ammonium hydroxide (TBAH), until a pH of 8 was obtained, and then dried at 70 °C. A fixed amount of acetone was added to dissolve the material and an appropriate amount of benzyl bromide was added, based on the amount of TBAH used to react with volatile fatty acids. After the sample preparation was completed, gas chromatography was performed to analyze the content of these acids. The silage quality was evaluated by Fleig's score, which was calculated from the equivalent percentage of acetic acid, butyric acid and lactic acid.. A score of 40 and less indicates that the silage quality is unacceptable; a score of 40-60 indicates that the quality is acceptable; a score of 60-80 represents good silage; and a score of above 80 means very good quality (Chen *et al*. 2000). Since the experimental system is sealed with no leakage, the dry matter content of silage divided by the dry matter content of it before ensiling can be used to calculate the dry matter recovery.

Results and discussion

I. Isolation and screening of bacterial strains

A total of 60 bacterial strains were isolated from different sources and successive culture was performed to retain strains with good activity. A total of 36 gram-positive strains were obtained after microscopy examination, which were used for the salinity tolerance and acidity tolerance experiments.

(1) Salinity tolerance experiment results: after culture for 48 h, there were 7 strains with pH values under 3.8

in condition of 0% salinity, 13 strains with pH value under 4 in condition of 5% salinity, and 4 strains with pH value under 5 in 10% salinity culture medium. (2) Acidity tolerance experiment results: when the initial pH of the culture medium was 6.05, after 24 h cultivation, 8 strains had pH value under 4 and after 72 h of culture, 12 strains had pH value less than 4; when the initial pH of the culture medium was 4.5, there were 5 strains with pH value below 3.5 after 48 h of culture, and 11 strains reached the same acidity level after 72 h of culture; in a low pH environment (initial pH of 3.5), only two strains (ST12 and WS) had a pH value lower than 3.3 after 48 h of culture and only 9 strains had the same level after 72 h of culture.

The 9 strains selected from the low pH condition and the 13 strains selected from the 5% salinity (a total of 22 strains) were used for the initial inoculant screen.

II. Alfalfa silage

(i) Initial screening of bacterial strains

The moisture content of the un-wilted alfalfa was 72.3%. When the silage were opened after 8 weeks, the pH of the control treatment was 4.86, whereas the pH of the commercial inoculant (ECO) was 4.64 and the pH values of the silage inoculated with our isolated strains were between 4.61 and 4.81, which was lower than control. Among the isolated strains, the treatment of ST15 had the lowest pH value of 4.61.

After wilting, the moisture content of the material was 58.3%. The pH changes after different period of ensiling, which presented a decreasing trend, were shown in Figure 1. Earlier than 2 weeks into the ensiling period, the performances of the various inoculated groups varied and were not significantly better than that of the control group. At week 4 in the ensiling period, some treated groups caused a greater reduction in pH. Among these groups, the commercial strain and 10 isolated strains had a pH value that was significantly lower than the control group (pH 5.45). In week 8 of the ensiling period, there was limited pH reduction in the control group, whereas the pH values of the inoculated treatment groups were all lower than that of the control group. Among these groups, inoculation with strain ST12 resulted in the lowest pH (4.88). Regardless of wilting, alfalfa silage showed significant gas production, but inoculation decreased gas production. Among the inoculants, D41 resulted in the greatest improvement in gas production.

In summary, from the above results, the strains ST15, ST12, and D41 were selected for the development of microbial inoculants for silage and sent for identification at the Food Industry Research and Development Institute. These strains were also used for further investigation of the effects of microbial inoculants on silage fermentation and nutrient composition.

(ii) Identification of microbial inoculants

After identification by the Food Industry Research and Development Institute, it was found that strain 41 was *Lactobacillus casei* and strains ST12 and ST15 were both *Lactobacillus plantarum* subsp. *plantarum*.

(iii) Inoculation testing

The raw material for this experiment was wilted alfalfa, which had a moisture content of 56.9%, a protein content of 12.9%, a neutral detergent fiber content (NDF) of 34.7%, and an acid detergent fiber (ADF) content of 30.7% before ensiling.

After 12 weeks of ensiling, the ADF content was increased, whereas there were no significant changes in crude protein and NDF contents, and no significant differences between the various

treatments (Table 1).

The dry matter content and dry matter recovery after ensiling are shown in Table 2. The dry matter content was between 38.9% and 42.1%, and the changes were small, whereas the dry matter recovery exceeded 90%. The silage fermentation results are shown in Table 3. The pH value in the control group decreased during the ensiling period.

At weeks 4, 8, and 12 of the ensiling period, the pH values were 5.51, 5.34, and 4.93, respectively, and the pH of the inoculation treatment groups were all lower than that of the control. Prior to week 8, the differences between the various inoculation treatment groups and the control group were relatively large. At week 12, the differences between the inoculation treatment groups and the control group were decreased, which indicated that a faster fermentation rate occurred after inoculation treatment. Among the inoculation treatment groups, D41 produced the fastest

Table 1. Chemical compositions of wilted alfalfa (pre-ensiling) and different treated alfalfa silage

	Treatment*	Crude protein	Neutral detergent fiber	Acid detergent fiber
		----------%		
Pre-ensiling		12.9	45.2	30.4
Ensiled 12 weeks	Control	13.3	45.1	36.3
	ECO	12.7	46.1	36.8
	D41	12.7	47.4	38.9
	ST12	13.3	46.2	35.7
	ST15	12.5	47.1	37.4

*: Control, without inoculation; ECO, inoculated with commercial product Ecosyl; D41, inoculated with isolated strain D41 (*L. casei*); ST12, inoculated with isolated strain ST12 (*L. plantarum*); ST15, inoculated with isolated strain ST15 (*L. plantarum*)

Table 2. Dry matter content and dry matter recovery of alfalfa silage inoculated with different strains of lactic acid bacteria.

Treatment*	Dry matter content(%)			Dry matter recovery(%)		
	4W	8W	12W	4W	8W	12W
Control	40.5^{ab}	40.4^{b}	40.8^{a}	93.9^{a}	93.6^{b}	94.5^{a}
ECO	40.8^{a}	40.4^{b}	43.0^{a}	94.6^{a}	93.7^{b}	95.0^{a}
D41	40.9^{a}	42.1^{a}	38.9^{b}	94.9^{a}	97.6^{a}	90.2^{b}
ST12	39.7^{b}	40.8^{b}	41.0^{a}	92.1^{b}	94.6^{a}	95.1^{a}
ST15	40.4^{ab}	40.5^{b}	39.4^{b}	93.7^{a}	94.0^{a}	91.3^{b}

$^{a, b, c}$ Means in the same section and the same column with different superscripts differ significantly ($P<0.05$).
* : Same as that in Table 1.

Table 3. Effect of inoculation of lactic acid bacteria on pH value, volatile fatty acids, and Fleig's score of alfalfa silage

Ensiling period	Inoculant*	pH	Acetic acid	Propionic acid	Butyric acid	Lactic acid	Flieg's score	A/L
week			----------% DW----------					
4	Control	5.51^{a}	0.29^{bc}	0.00^{a}	0.00^{b}	0.34^{c}	68^{c}	0.85^{a}
	ECO	5.17^{b}	0.30^{bc}	0.00^{a}	0.04^{a}	0.60^{bc}	69^{c}	0.49^{b}
	D41	4.23^{c}	0.37^{b}	0.00^{a}	0.00^{b}	2.22^{a}	100^{a}	0.17^{c}
	ST12	5.13^{b}	0.22^{c}	0.00^{a}	0.00^{b}	0.64^{bc}	90^{ab}	0.35^{b}
	ST15	5.04^{b}	0.50^{a}	0.00^{a}	0.00^{b}	1.10^{b}	84^{b}	0.46^{b}
8	Control	5.34^{a}	0.88^{a}	0.22^{a}	0.00^{b}	0.26^{c}	50^{b}	3.36^{a}
	ECO	5.09^{a}	0.30^{a}	0.14^{a}	0.00^{b}	0.37^{c}	51^{b}	2.35^{a}
	D41	4.31^{c}	0.45^{b}	0.00^{b}	0.00^{b}	2.24^{a}	98^{a}	0.20^{b}
	ST12	4.80^{b}	0.29^{b}	0.00^{b}	0.00^{b}	1.14^{b}	95^{a}	0.26^{b}
	ST15	4.88^{b}	0.30^{b}	0.00^{b}	0.04^{a}	0.88^{b}	79^{a}	0.34^{b}
12	Control	4.93^{a}	1.67^{a}	0.30^{a}	0.10^{a}	0.69^{b}	38^{b}	2.49^{c}
	ECO	4.70^{b}	0.64^{b}	0.00^{b}	0.00^{b}	1.71^{a}	89^{a}	0.37^{b}
	D41	4.82^{c}	0.87^{b}	0.00^{b}	0.00^{b}	1.71^{a}	82^{a}	0.51^{b}
	ST12	4.67^{b}	0.68^{b}	0.00^{b}	0.00^{b}	1.83^{a}	89^{a}	0.38^{b}
	ST15	4.69^{b}	0.67^{b}	0.00^{b}	0.00^{b}	1.82^{a}	88^{a}	0.38^{b}

$^{a, b, c}$ Means in the same section and the same column with different superscripts are different significantly ($P<0.05$).
* : Same as that in Table 1.

response: at week 4 and week 8, the pH value was 4.23 and 4.31, respectively, which were the lowest values among the various groups . However, at week 12, the ST12 resulted in the lowest pH value of 4.67. Inoculation treatment significantly improved the pH value of silage. The fermentation acid products of alfalfa silage consisted mainly of lactic acid and acetic acid. Acid production increased with ensiling period, However, the rate of increase in acetic acid production was higher than that of lactic acid in the control group, which resulted in an increase in the acetic acid/lactic acid ratio and a decrease in the silage score. The evaluation score decreased from 68 points at week 4 to 38 points at week 12; thus, good-quality silage was degraded to unacceptable silage. The lactic acid content in the inoculation treatment groups was significantly higher than that in the control group, and the acetic acid/lactic acid ratio was significantly lower than that in the control group. This showed that inoculation treatment can promote lactic acid fermentation in alfalfa silage. Simultaneously, the evaluation score of silage at week 12 in the inoculation treatment groups was still between 82 and 89 points, which demonstrated the superior quality of the silage. For the bacterial strains, our isolated strains resulted in better fermentation and silage evaluation scores at weeks 4 and 8 compared with the commercial strain. This showed that our isolated

strains promoted fermentation more rapidly compared with the commercial strain.

Conclusion

The results of the alfalfa inoculation experiments by Filya *et al*. (2007) demonstrated that the environmental adaptability and activity of different bacterial strains varied and the acid production in silage fermentation after inoculation was also different. Homofermentative lactic acid bacteria will completely convert the sugars to lactic acid and promote the rapid synthesis of lactic acid in silage, whereas heterofermentative lactic acid bacteria will synthesize acetic acid in addition to lactic acid. In this study, the rate of acetic acid synthesis was higher than that of lactic acid synthesis in the control group at the late fermentation stage and further study is required to see whether this is related to the above study. This study also showed that lactic acid bacteria inoculation is beneficial for alfalfa silage fermentation and the performances of our isolated strains were better than those of commercial strains. This may be a result of the screening by tolerance and material characteristics, but may also be related to better adaptation to the growth environment.

The results of this study showed that lactic acid bacteria isolated from diverse sources can be developed into silage inoculants for the fermentation of legume forage. The isolated laboratory strains show characteristics of adaptation to the local environment and, in the future, silage material characteristics could be used to screen different inoculation strains

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